

**PATENT**

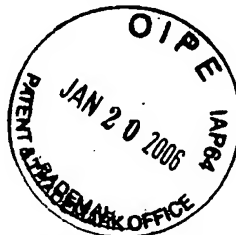
**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

IN RE APPLICATION OF:  
YAMAZAKI, *et al.*  
APPLICATION No.: 10/661,790  
FILED: September 11, 2003  
FOR: **MEMBRANE BASED ASSAYS**

EXAMINER: FOSTER, CHRISTINE E.  
ART UNIT: 1641  
CONF. NO: 9161

**Declaration Under 37 C.F.R. §1.132**

Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450



Sir:

I, Victoria Yamazaki, declare and state that:

1. I was employed by Synamem Corporation and Proteomic Systems, Inc. from 2001–2004. During this time I held the positions of Senior Scientist, Director of Membrane Biology, VP, Biological R&D, and Secretary of the Corporation.
2. I received my Ph.D. from Stanford University in 1999 in Biophysics.
3. I have been active in the field of biochemistry and biophysics for over 18 years. Two key areas of my technical expertise are biophysics and biochemistry.
4. I am a named co-inventor of the subject matter embodied claims 1-41 of the above-referenced application.
5. On December 16, 2004, a manuscript was submitted for publication in the *Journal of the American Chemical Society* based on experiments and studies which I performed, directed and/or analyzed concerning the effects of ligand binding on fluidity of a supported membrane microarray (copy of the published article enclosed herewith).

6. As described in the article, a lipid microarray was prepared using the lipids DMPC (98.75%) and NBD-PG (1 mol %). The lipid array also included 0.25 mol % of a GPI-linked form of Intercellular Cellular Adhesion Molecule 1 (ICAM-1).

7. The lipid microarray was exposed to increasing concentrations of a phycoerythrin-conjugated anti-ICAM-1 antibody having specific binding affinity for ICAM-1. Before and after binding, the fluidity of the lipid microarray (referred to in the article as "membrane mobility") was measured by fluorescence recovery after photobleaching (FRAP).

8. As seen in the data presented in the article, a decrease in lipid fluidity was found upon binding with the anti-ICAM1 antibody (see Fig. 2).

I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of title 18 of the United States Codes and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: \_\_\_\_\_

Signed: \_\_\_\_\_  
Victoria Yamazaki

## Lipid Mobility and Molecular Binding in Fluid Lipid Membranes

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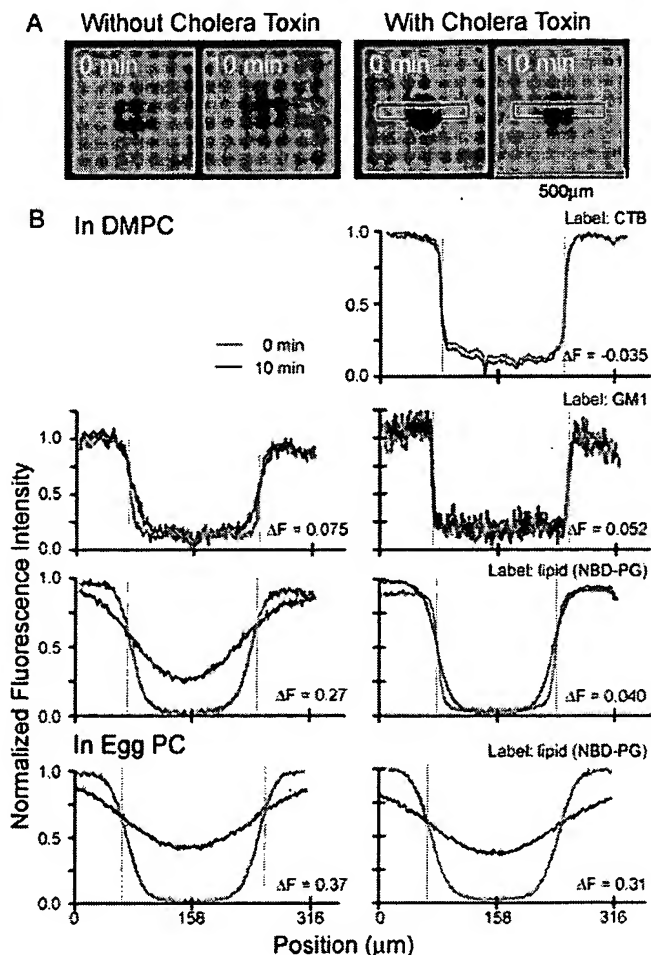
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Innumerable biochemical events, ranging from intercellular signaling to viral infection, involve receptor–ligand engagement on the cell membrane surface. It is becoming increasingly evident that spatial rearrangement of receptors and signaling molecules within the fluid membrane environment is a broadly significant aspect of these processes. Polyvalent ligands, for example, induce co-localization of their target receptors, thus encoding collective properties that are appreciatively different from individual binding events.<sup>1</sup> Correspondingly, the ability of target receptors to move and adopt complementary configurations is intimately associated with the overall affinity of the molecular recognition event.<sup>1–3</sup> In other examples, such as G-protein coupled receptor (GPCR) and integrin signaling, ligand binding triggers a conformational change in the receptor protein which, in turn, alters its association state with other membrane-localized signaling molecules.<sup>4,5</sup> In each case, changes in the organization and mobility of membrane components occur in conjunction with signaling and recognition events.

Here, we examine the mobility of nonparticipating background lipid in conjunction with ligand binding membrane-associated receptors in a fluid lipid bilayer membrane. Despite the fact that the background lipid is not directly involved in the ligand binding interaction, binding-induced mobility changes are clearly discernible for the two systems studied: cholera toxin binding membrane-associated monosialoganglioside  $G_{M1}$  and antibody binding to a glycanphosphatidylinositol (GPI)-linked form of intercellular adhesion molecule (ICAM-1). A useful corollary of this third-party effect is that binding can be detected without labeling the ligand or the receptor of interest.

Experiments were performed using supported membrane microarrays.<sup>6–9</sup> Membrane arrays were assembled on silica substrates, which had been photolithographically patterned with chrome grids. The chrome creates surface barriers that isolate the individual membrane corrals. Robotic direct dispensing methods with Cartesian MicroSys model 4100-2SQ were employed to deposit 40 nL droplets of vesicle suspension into the prepatterned 500 × 500  $\mu\text{m}$  corrals. Vesicle fusion occurs within seconds of deposition, forming fluid-supported membranes that continuously fill each corral (Figure 1A). Mobility of fluorescently labeled components was monitored by fluorescence recovery after photobleaching (FRAP). FRAP measurements were performed using a  $\sim 100 \mu\text{m}$  diameter bleaching spot and a 60 s bleach exposure time. For this spot size, observations of diffusive recovery were made several minutes after the bleach exposure.

Results characterizing molecular mobility within the supported membrane during cholera toxin binding are summarized in Figure 1. Cholera toxin, which is naturally secreted by *Vibrio cholerae*, exists as a hexamer involving two different types of subunits in an  $AB_5$  configuration. The B subunits (CTB) organize into pentamers with each subunit specifically binding the ganglioside  $G_{M1}$  pen-



**Figure 1.** (A) Representative FRAP experiments on a pair of 500 × 500  $\mu\text{m}$  membrane corrals containing unlabeled ganglioside  $G_{M1}$  (0.25 mol %) with background lipids consisting of DMPC (98.75 mol %) and NBD-PG (1 mol %). Experiments were performed before and after exposure to CTB ( $1.40 \times 10^{-7}$  M), as labeled. The 0 min images depict the photobleached spots immediately after exposure to bleaching light. Images taken 10 min later reveal the extent of diffusive mixing. (B) Quantitative traces of fluorescence intensity across the bleach spot at 0 and 10 min for a series of FRAP experiments probing the change in mobility of each component upon CTB binding, as labeled. The parameter,  $\Delta F$ , represents the linearly integrated and normalized difference between before and after fluorescence traces. A value of 0 indicates no diffusion, and a value of 1 indicates complete recovery.

tasaccharide headgroup.<sup>10,11</sup> Binding of CTB to  $G_{M1}$  containing supported membranes is readily confirmed using fluorescently labeled CTB (Alexa Fluor 594 conjugate) (Supporting Information).

FRAP mobility measurements of labeled CTB, labeled  $G_{M1}$ , and labeled lipid (NBD-PG) are summarized in Figure 1B. Observations of labeled CTB indicate that it is relatively immobile when bound to supported membranes. The large size, potential for oligomer-

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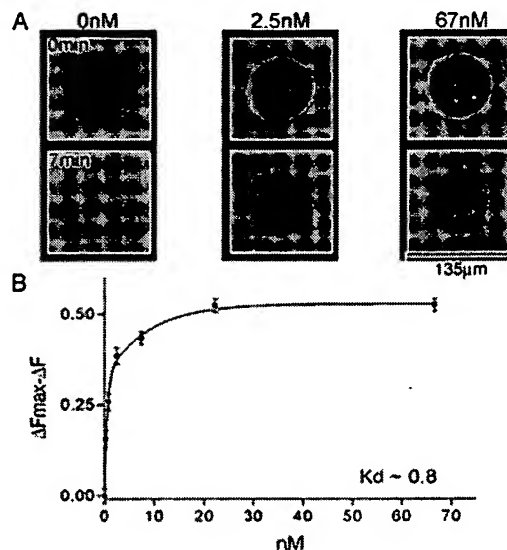
ization, and multivalent binding of CTB likely contribute to this reduced mobility. A corresponding set of experiments, utilizing labeled  $G_{M1}$  (BODIPY FL C5) and unlabeled CTB, was performed to characterize the mobility of  $G_{M1}$  during CTB binding. Before exposure to CTB, labeled  $G_{M1}$  exhibits lateral diffusion, though somewhat attenuated relative to other lipids, perhaps as a result of slight aggregation (Figure 1B). After CTB binding, a substantial reduction in the diffusion rate of labeled  $G_{M1}$  (now complexed with CTB) is observed.

A most interesting feature of these experiments is revealed when the mobility of the lipid probe (NBD-PG) is monitored during CTB- $G_{M1}$  binding. Despite the fact that this lipid does not participate in the binding interaction, its mobility is markedly affected by CTB- $G_{M1}$  binding. FRAP experiments on the 1 mol % NBD-PG in DMPC/ $G_{M1}$  (98.75/0.25 mol %) membranes reveal a drastic reduction in mobility in conjunction with CTB binding (Figure 1B). Similar experiments, performed using egg-PC (a natural mixture of PCs containing ~50% unsaturated fatty acids) instead of the saturated DMPC, do not show a reduction in NBD-PG mobility associated with CTB- $G_{M1}$  binding. The independence of NBD-PG mobility from CTB- $G_{M1}$  binding in egg-PC membranes confirms that NBD-PG has no intrinsic interaction with CTB or  $G_{M1}$ . An important difference between egg-PC and DMPC membranes is the gel-fluid transition temperature of DMPC (23 °C), which is much higher than that of egg-PC (<10 °C). Proximity to a gel-fluid transition contributes to the mobility effect we observe in the DMPC system (Supporting Information).

We therefore suggest that protein binding modulates the gel-fluid transition temperature of the membrane. As a corollary of this, one can expect the miscibility phase transition temperature of a mixed membrane to be similarly modulated by protein binding. Preliminary studies indicate this is so.

To test the generality of using lipid mobility as a measure of receptor-ligand binding, antibody binding to the cell surface adhesion molecule, ICAM-1, was examined. A GPI-linked form of ICAM, which is known to be biologically functional in the supported membrane configuration,<sup>12</sup> was used for this study. This protein was expressed in CHO cells, purified, and reconstituted into preformed lipid vesicles (99% DMPC, 1% NBD-PG) by detergent dialysis (Supporting Information). A phycoerythrin-conjugated anti-ICAM-1 antibody (BD Biosciences) was utilized for direct fluorescence observation of the protein. FRAP images and compiled results from the lipid mobility assay (Figure 2) reveal an effective  $K_d$  of ~0.8 nM for the antibody-ICAM-1 interaction, which is comparable to the  $K_d$  of ~3.8 nM obtained from direct fluorescence measurements of the labeled antibody (Supporting Information).

A useful consequence of the mobility effects described here is that traces of fluorescent probe lipid, doped into the background of the membrane, can be utilized to detect binding of unlabeled ligands to unmodified membrane targets. Low concentrations of target (0.25 mol %) can trigger substantial mobility changes. The  $G_{M1}$  target concentration used in these experiments is 20-fold lower than the 5 mol %  $G_{M1}$  Kuziemko et al. report as the minimum required for analyzable kinetic data using a Biacore surface plasmon resonance system.<sup>13</sup> With use of a laser, diffusion measurements could be executed rapidly (seconds), enabling acquisition of kinetic



**Figure 2.** (A) Corals of chips containing 0.25 mol % ICAM-1 in 98.75% DMPC with 1 mol % NBD-PG were exposed to increasing concentrations of anti-ICAM-1 antibody (BD Biosciences). FRAP was performed on at least 4 corals for each concentration of antibody. (B) The average  $\Delta F$  (as described in Figure 1) for each antibody concentration was subtracted from the average maximal  $\Delta F$  ( $\Delta F_{\max}$ ), which was obtained from corals exposed to no antibody.  $K_d$  was determined with Prism 3.03.

binding data by lipid mobility analysis. This methodology may be extended to studies of fully transmembrane proteins, such as the GPCRs, by using polymeric layers on the supporting substrate to facilitate lateral mobility of the protein within the membrane.

**Acknowledgment.** This work was supported by NSF SBIR Grant Award DMI-0320515. We gratefully acknowledge Luat Nyugen's superb technical support, and Nick Ulman and Steve Sundberg for fabrication of the substrates and image analysis. All experiments were performed at Synamem labs.

**Supporting Information Available:** Binding affinity on a chip, lipid mobility at 22 and 30 °C, and fluorescent anti-ICAM-1 antibody affinity (pdf). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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JA042430L